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Preferential M2 macrophages contribute to fibrosis in IgG4-related dacryoadenitis and sialoadenitis, so-called Mikulicz's disease

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Abstract IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) is characterized by bilateral swelling of glandular tissues with extensive fibrosis, and is immunologically considered a Th2-predominant disease. Recent studies reported that alternatively activated (M2) macrophages enhanced Th2 immune responses and fibrosis by production of pro-fibrotic factors (IL-10, IL-13 and CCL18). Therefore, we examined the association between M2 macrophages and fibrosis in submandibular glands from 7 patients with IgG4-DS, 10 patients with chronic sialoadenitis, 10 patients with Sjögren's syndrome, and 10 healthy subjects. The number of M2 macrophages in SMGs from patients with IgG4-DS was also significantly higher than in the other groups. Double immunofluorescence staining showed that IL-10 and CCL18 expression co-localized with M2 macrophage-marker (CD163). Furthermore, the SMG fibrosis score was positively correlated with the frequency of M2 macrophages in only IgG4-DS. These results indicate that IL-10 and CCL18 secreted by preferential M2 macrophages possibly play a key role in the development of severe fibrosis in IgG4-DS.

Abbreviations: MD, Mikulicz's disease; SMG, Submandibular gland; LG, lacrimal gland; SS, Sjögren's syndrome; IgG4-RD, IgG4-related disease; IgG4-DS, IgG4-related dacryoadenitis and sialoadenitis; Th2, helper T type 2; eGC, ectopic germinal center; OSCC, oral squamous cell carcinoma; CS, chronic sialoadenitis; MT, Masson's trichrome; Treg, regulatory T cell.

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1. Introduction

Mikulicz's disease (MD), first reported by Mikulicz in 1888 [1], is characterized by firm swelling of submandibular glands (SMGs) and lacrimal glands (LGs), and has been considered to be a subtype of Sjögren's syndrome (SS) because of these histopathological similarities [2]. However, Yamamoto et al. reported that patients diagnosed with MD also had high serum levels of IgG4 and marked infiltration of IgG4-positive plasma cells in salivary glands [3]. Moreover, several reports also demonstrated that these findings were accompanied by autoimmune pancreatitis (AIP) [4], sclerosing cholangitis (SC) [5], tubulointerstitial nephritis (TIN) [6], interstitial pneumonia [7], Hashimoto's thyroiditis [8] and Küttner tumor [9]. These diseases are now collectively called "IgG4-related disease (IgG4-RD)" and we have described this concept and provided up-to-date information regarding this emerging disease entity in a recent review [10]. Furthermore, recent studies have also referred to MD as IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) [11].

Regarding the immunological aspect of IgG4-RD, it is well known that IgG4 is induced by T helper type 2 cell (Th2) cytokines such as interleukin (IL)-4 and IL-13. We previously reported that analysis of peripheral $CD4^+$ Th cells from patients with IgG4-DS revealed a deviation in the Th1/Th2 balance favoring Th2 [12] and that Th2 cells play a key role in the production of IgG4 and formation of ectopic germinal centers (eGCs) [13,14]. In addition, Watanabe et al. reported that abnormal immune responses might enhance the Th2 response via Toll-like receptors expressed by macrophages, contributing to the immunopathology of IgG4-RD [15]. Recent studies reported that rituximab targeting CD20 plasma cells appeared to be an effective treatment strategy for IgG4-RD, but plasmablasts lack surface expression of CD20 and thus demonstrate a resistance to direct depletion by rituximab and IgG4-positive plasmablast. Macrophage might play an effective role in IgG4 production produced by IgG4-positive plasmablast. Recently, innate immune system such as macrophages has received a lot of attention to the initiation of IgG4-RD [16]. At least two distinct subtypes of macrophages have been identified; the classically activated (M1) macrophage stimulated by Th1 responses and the alternatively activated (M2) macrophage stimulated by Th2 responses. M2 macrophages contribute to angiogenesis, suppression of adaptive immunity, and wound healing and fibrosis [17,18]. Histologically, the critical features of IgG4-RD are severe fibrosis with dense lymphoplasmacytic infiltration of the salivary glands and other lesion [19]. However, to our knowledge, no published reports have investigated the mechanism promoting severe fibrosis in IgG4-RD. In this study, we examined the distribution of macrophage subsets and the expression of pro-fibrotic factors in salivary glands to clarify the contribution of macrophages to the pathogenesis of IgG4-DS.

2. Material and methods

2.1. Patients

SMG samples were collected from 7 patients with IgG4-DS (five men and two women; mean age ± standard deviation

Table 1 Clinical characteristics of 7 patients with IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS).

No.	Age	Sex	Disease duration	Complications	Swollen glands					Complaint		Histological findings		Serological test							
					LG	PG	SMG	SLG	PLG	LSG	Dry eye	Dry mouth	IgG4/IgG (%)	$IgG4^+$ cells (/HPF)	RF (U/ml)	ANA	IgG (mg/dl)	IgG4 (mg/dl)	IgA (mg/dl)	IgE (IU/ml)	IgM (mg/dl)	Anti-SS-A (U/ml)	Anti-SS-B (U/ml)
1	58	F	6 M	–	–	–	+	+	–	–	–	–	***73.0***	***28***	4	***80***	1188	***151***	193	***178***	56	–	–
2	68	F	9 M	AIP, TIN	–	–	+	+	–	+	–	–	***52.3***	***42***	5	***160***	***6758***	***1500***	78	13	81	–	–
3	39	M	2 Y	–	+	–	+	–	–	+	–	–	***64.2***	***77***	5	–	1534	***188***	170	***1619***	99	–	–
4	69	M	3 M	HT	+	–	+	+	–	+	–	–	***61.2***	***32***	ND	–	***1662***	***458***	97	60	79	–	–
5	74	M	4 M	AIP, Prostatitis	+	–	+	+	–	+	–	–	***50.0***	***85***	ND	40	***4217***	***524***	177	29	60	–	–
6	55	M	3 Y	AIP, IP	+	–	+	+	–	–	–	–	***70.0***	***18***	4	–	***2092***	***510***	148	ND	70	–	–
7	69	M	4 M	AIP	–	–	+	+	–	+	–	–	***63.2***	***79***	ND	–	***1675***	***484***	229	***283***	44	–	–

Abbreviations: LG, lachrymal gland; PG, parotid gland; SMG, submandibular gland; SLG, sublingual gland; PLG, palatine gland; LSG, labial salivary gland; TIN, tubulointerstitial nephritis; HT, higher tension; AIP, autoimmune pancreatitis; IP, interstitial pneumonitis; –, negative; ND, not done; bold italic means higher than normal values.

(SD), 61.7 ± 12.1 years), 10 patients with chronic sialoadenitis (CS) caused by sialolith (five men and five women; 51.5 ± 17.2 years), 10 patients with SS (five men and five women; 61.5 ± 14.9 years), and 10 patients with oral squamous cell carcinoma (OSCC) as a control group (five men and five women; 58.4 ± 16.3 years) who were referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital between 2010 and 2013. Patients underwent the following procedures: (1) open SMG biopsies for IgG4-DS and SS patients as described by Moriyama et al. [20]; (2) submandibulectomy for CS patients; and (3) neck dissection for OSCC patients. SMGs from OSCC patients were histologically normal and had no clinical evidence of metastasis. IgG4-DS was diagnosed according to both the "Comprehensive diagnostic criteria for IgG4-related disease" [10] and "Diagnostic criteria for IgG4-related Mikulicz's disease" [21]. The clinical and serological characteristics of the 7 patients with IgG4-DS are summarized in Table 1. SS was diagnosed according to both the Research Committee on SS of the Ministry of Health and Welfare of the Japanese Government (1999) [22] and the American–European Consensus Group criteria for SS [23]. Each patient showed objective evidence of salivary gland involvement based on the presence of subjective xerostomia and a decreased salivary flow rate, abnormal findings on parotid sialography and focal lymphocytic infiltrates in the labial salivary glands. There was no documented history of treatment with steroids, infection with HIV, HTLV-1, hepatitis B virus, or hepatitis C virus infection, sarcoidosis or any other immunodepressants in any of the patients. None of the patients had evidence of malignant lymphoma at the time of the study. The comparison of clinical and serological characteristics between the patients included in this study with SS and IgG4-DS is summarized in Table 2.

This study design was approved by the Ethics Committee of Kyushu University, Japan, and written informed consent was obtained from all of the patients and healthy controls (IRB serial number: 25-287).

Table 2 Comparison of clinical and serological findings between primary Sjögren's syndrome and IgG4-DS.

	SS (n = 10)	IgG4-DS (n = 7)
Mean age (years)	61.5 ± 14.9	61.7 ± 12.1
Men:women	5:5	5:2
Frequency of elevated serum IgG	60.0% (6/10)	71.4% (5/7)
<Mean ± SD (mg/dL)>	<2135.3 ± 757.0>	<2732.3 ± 2037.4>
Frequency of elevated serum IgG4	ND	100.0% (7/7)
<Mean ± SD (mg/dL)>	<ND>	<545.0 ± 448.6>
Frequency of elevated ANA	100.0% (10/10)	42.9% (3/7)
Anti-SS-A/Ro	100.0% (10/10)	0.0% (0/7)
Anti-SS-B/La	40.0% (4/10)	0.0% (0/7)
Frequency of other organs diagnosed with IgG4-RD	None	Pancreas: 57.1% (4/7) Kidney: 14.3% (1/7) Lung: 14.3% (1/7) Prostate: 14.3% (1/7)

Abbreviations: SS, Sjögren's syndrome; IgG4-RD, IgG4-related disease. Data are presented as the mean ± SD.

2.2. Immunohistochemical analysis

For immunohistochemical analysis, 4-μm formalin-fixed, paraffin-embedded sections were prepared and stained with a conventional avidin–biotin complex technique as previously described [13]. Anti-CD68 (catalog # ab955; Abcam, Cambridge, MA, USA) and CD163 (catalog # NCL-CD163; Leica Biosystems, Nussloch GmbH, Germany) mouse monoclonal antibodies were used to analyze the protein expression of CD68 and CD163, respectively. Anti-CCL18 (catalog # ab104867; Abcam), IL-10 (catalog # ab34843; Abcam) and IL-13 (catalog # HPA042421; Atlas Antibodies AB, Stockholm, Sweden) rabbit polyclonal antibodies were used to analyze the protein expression of CCL18, IL-10 and IL-13, respectively. Tissue sections were sequentially incubated with primary antibodies for 2.5 h then with biotinylated anti-mouse IgG and anti-rabbit IgG secondary antibodies (Vector Laboratories, Burlingame, CA, US), avidin–biotin–horseradish peroxidase complex (Vector Laboratories), and 3,3′-diaminobenzidine (Vector Laboratories). Mayer's hematoxylin was used for counterstaining. Photomicrographs were obtained using a light microscope equipped with a digital camera (BZ-9000 series; Keyence, Tokyo, Japan).

2.3. RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was prepared from whole LSGs and SMGs by the acidified guanidinium–phenol–chloroform method. One microgram of total RNA was used for the synthesis of cDNA. Briefly, RNA was incubated for 1 h at 42 °C with 20 U of RNase inhibitor (Promega, Madison, WI, USA), 0.5 μg of oligo-1218 (Pharmacia, Uppsala, Sweden), 0.5 mM of each deoxyribonucleotide triphosphate (dNTP) (Pharmacia), 10 mM of dithiothreitol (DTT), and 100 U of RNA reverse transcriptase (Life Technologies, Gaithersburg, MD, USA).

2.4. Quantitative estimation of mRNA by real-time PCR

The mRNA levels of the cytokines and chemokines were analyzed quantitatively by real-time PCR using Light Cycler Fast Start DNA Master mix SYBR Green III (Roche Diagnostics, Mannheim, Germany) in a Light Cycler real-time PCR instrument (version 3.5; Roche Diagnostics). The cytokines and cell surface markers analyzed were CD68, CD163, CCL18, IL-10, and IL-13. The primer sequences used were as follows: β-actin (260 bp), forward 5′-GCA AAG ACC TGT ACG CCA AC-3′, reverse 5′-CTA GAA GCA TTT GCG GTG GA-3′; CD68 (199 bp), forward 5′-TCA GAA TGC ATC CCT TCG AG-3′, reverse 5′-GAT GAG AGG CAG CAA GAT GG-3′; CD163 (168 bp), forward 5′-TGA TTT CGG ACT TCT CTC TGG-3′, reverse 5′-ACT GGG CAG AGT GAA AGA TG-3′; CCL18

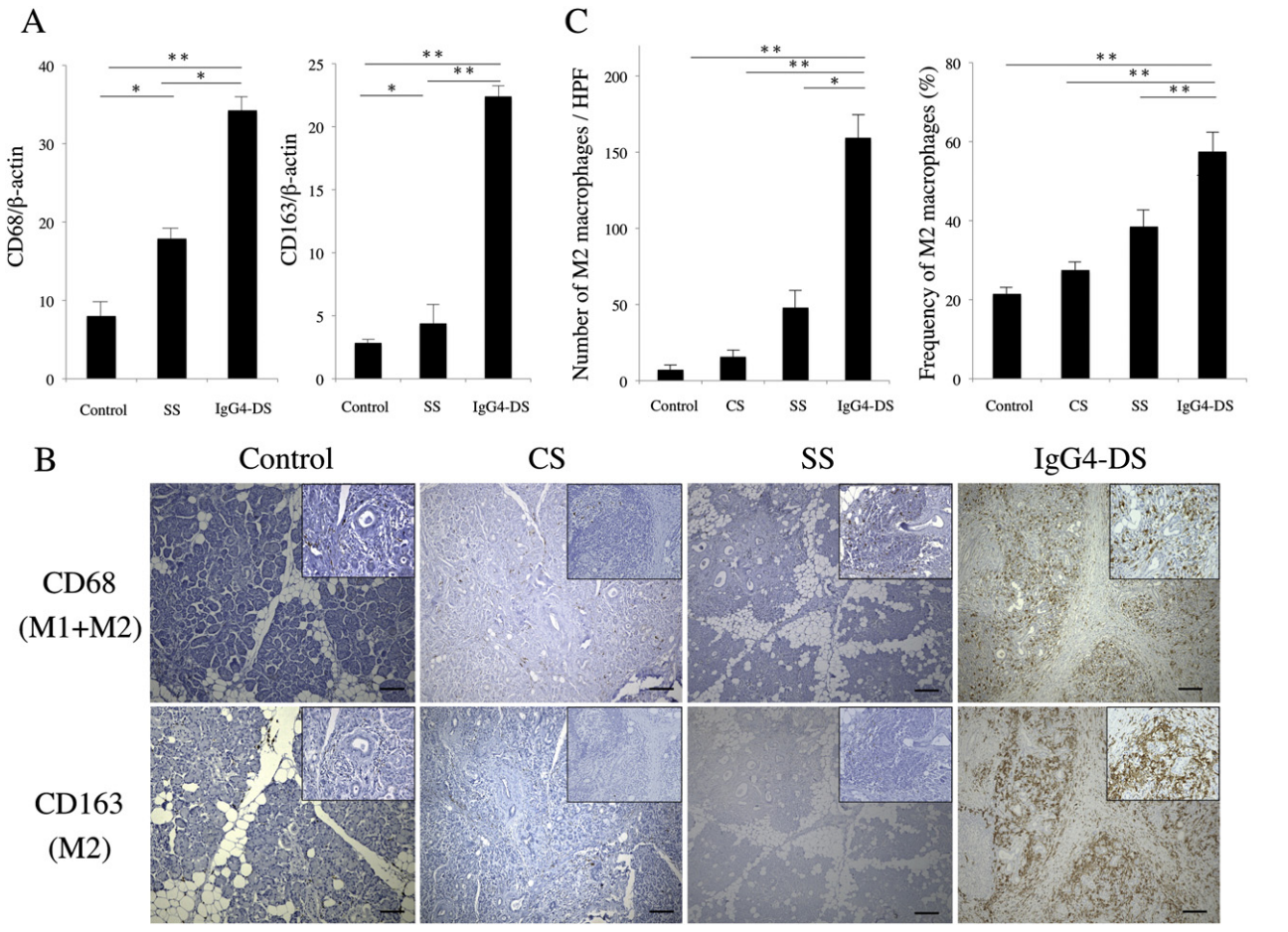

Figure 1 M1 and M2 macrophage localization in submandibular glands (SMGs). (A) mRNA expression levels of macrophage markers (CD68, M1 and M2 macrophages; CD163, M2 macrophages) were examined in SMGs from controls (n = 10), patients with Sjögren's syndrome (SS) (n = 10) and IgG4-related dacryoadenitis and sialoadenitis (IgG4-DS) (n = 7). Macrophage markers were quantitatively estimated as described in the Materials and methods section. Significant differences between groups were determined by the Mann–Whitney *U* test (*$p < 0.05$, **$p < 0.01$). (B) Distribution of M1 and M2 macrophages in SMGs from representative controls and patients with chronic sialoadenitis [33], SS and IgG4-DS. Counterstaining was performed with Mayer's hematoxylin (blue). Higher magnifications are displayed at the upper right. Scale bars, 100 μm. (C) Number and frequency of M2 macrophages per high-power field (HPF) were counted in 4-mm^2 sections from five different areas as described in the Materials and methods section. Statistically significant differences between groups were determined by Mann–Whitney *U* tests (*$p < 0.05$, **$p < 0.01$).

(187 bp), forward 5′-AGC TCT GCT GCC TCG TCT AT-3′, reverse 5′-CAG GCA TTC AGC TTC AGG TC-3′; IL-10 (144 bp), forward 5′-TGA GAA CCA AGA CCC AGA CA-3′, reverse 5′-AAG GCA TTC TTC ACC TGC TC-3′; and IL-13 (240 bp), forward 5′-GGT CAA CAT CAC CCA GAA CC-3′, reverse 5′-TTT ACA AAC TGG GCC ACC TC-3′. The relative mRNA level was calculated after normalizing to the housekeeping gene β-actin.

2.5. Double immunofluorescence analysis

For double immunofluorescence analysis, 4-μm formalin-fixed, paraffin-embedded sections were prepared and stained. Sections were incubated with the primary antibody, CCL18 (Abcam), IL-10 (Abcam), or IL-13 (Atlas Antibodies AB) at room temperature for 2 h after blocking with 1% BSA for 1 h, then incubated with secondary antibody (1:100 dilution of Alexa 488 USA) for 30 min. The sections were washed well, blocked with 1% BSA blocking buffer for 40 min, and incubated with the primary antibodies, CD163 (clone EDHu-1; AbD Serotec, Raleigh, NC, USA) at room temperature for 2 h. After incubation, the sections were incubated with secondary antibodies (1:100 dilution of Alexa 568) for 30 min at room temperature. Slides were mounted (VectaMount, Vector Laboratories) and kept in the dark. DAPI was used to stain nuclei. Images were taken using a Keyence microscope (BZ-9000 series), setting the background fluorescence level with the negative controls.

2.6. Evaluation of the severity of fibrosis

To evaluate fibrosis histologically, Masson's trichrome (MT) staining (Polysciences, Warrington, PA, USA) was performed.

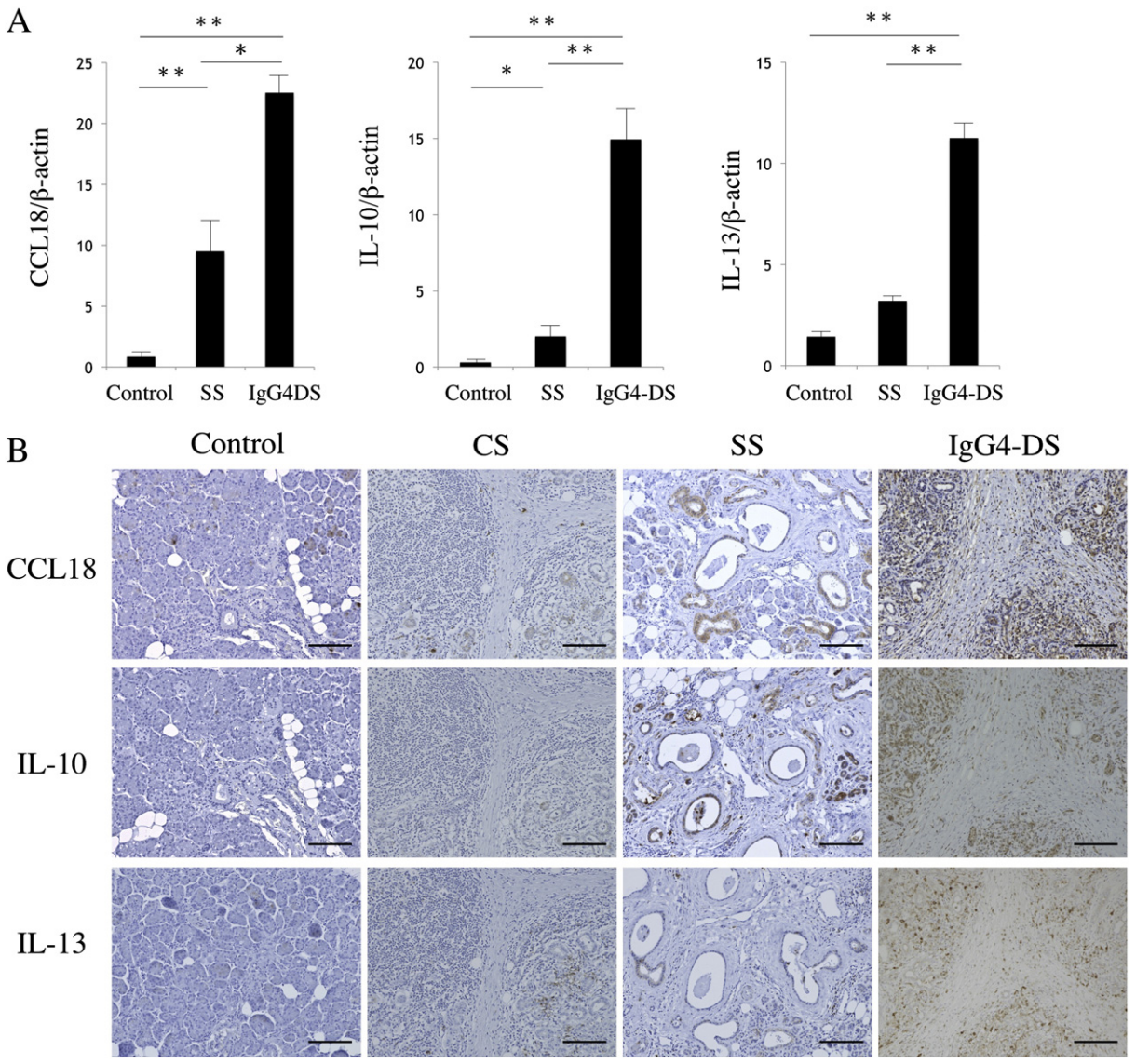

Figure 2 Localization of pro-fibrotic factors (CCL18, IL-10, and IL-13) in SMGs. (A) mRNA expression levels of pro-fibrotic factors in SMGs from controls (n = 10), patients with SS (n = 10) and IgG4-DS (n = 7). Expression levels of pro-fibrotic factors were estimated quantitatively as described in the Materials and methods section. Statistically significant differences between groups were determined by Mann–Whitney *U* tests (*$p < 0.05$, **$p < 0.01$). (B) Distribution of fibrosis factors in SMGs from representative controls and patients with CS, SS, and IgG4-DS. Counterstaining was performed with Mayer's hematoxylin (blue). Scale bars, 200 μm.

In short, 4-μm formalin-fixed, paraffin-embedded sections were prepared and stained. Connective and fibrosis tissues were selectively stained blue, whereas nuclei stained by Weigert's iron hematoxylin were dark brown to black and the cytoplasm was stained red. The fibrosis scores in SMGs were defined as the ratio of the fibrotic area (blue) to the whole stained area in a 4-mm^2 field of view, from five different areas.

2.7. Statistical analysis

The statistical significance of the differences between the groups was determined by the Mann–Whitney *U* test or Spearman's rank correlation as appropriate. All statistical analyses in this study were performed using JMP software, version 8 (SAS Institute, Cary, NC, USA). A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Expression of macrophage markers in SMGs

The mRNA levels of CD68 and CD163 in SMGs from SS and IgG4-DS patients were significantly higher than those in controls. Furthermore, the mRNA levels of CD68 and CD163 in IgG4-DS patients were significantly higher than those in SS

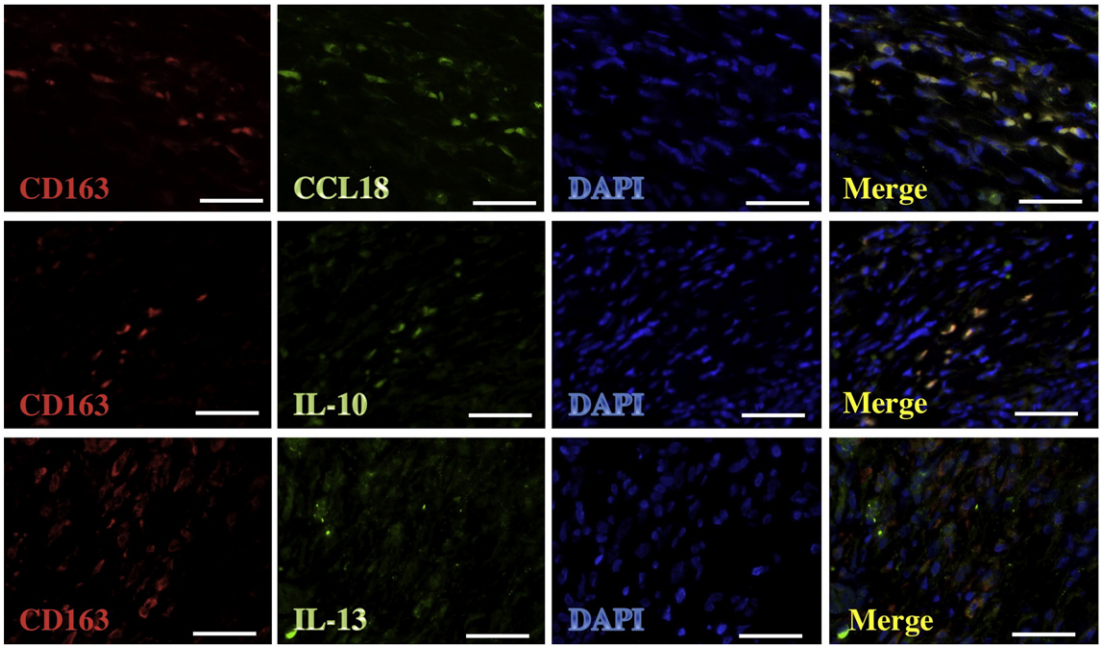

Figure 3 M2 macrophages produce pro-fibrotic factors in SMG in patients with IgG4-DS. Double immunofluorescence staining performed with CD163 (red), pro-fibrotic factors (CCL18, IL-10, and IL-13) [23], and DAPI for staining nuclei (blue) as described in the Materials and methods section. The images for CD163 and pro-fibrotic factors were merged (yellow). Scale bars, 50 μm.

patients (Fig. 1A). As specimens from CS patients were formalin-fixed and paraffin-embedded tissues, the mRNA levels in CS patients were not measured. The specimens were also examined after the immunohistochemical staining to evaluate the distribution of macrophages in SMGs. Expression of CD68 was weakly detected in/around areas of fibrosis or connective tissue in SMGs from controls, while it was strongly detected in/around areas of fibrosis in SS, CS and IgG4-DS patients. Expression of CD163 was not detected in controls, but was weakly detected in/around fibrotic lesions in SS and CS patients. Interestingly, expression of CD163 was strongly detected in/around areas of fibrosis in IgG4-DS patients (Fig. 1B). Moreover, the number of CD163-positive cells and the CD163/CD68 ratio in SMGs from IgG4-DS patients were significantly greater than those in the other groups (Fig. 1C).

3.2. Expression of pro-fibrotic factors in SMGs

As IL-10, IL-13, and CCL18 produced by macrophages promote fibrosis, we next compared the expression and distribution of these pro-fibrotic factors between controls and patients. The mRNA levels of CCL18, IL-10, and IL-13 in SMGs from SS and IgG4-DS patients were significantly greater than those in controls. Furthermore, the mRNA levels of CCL18, IL-10 and IL-13 in IgG4-DS patients were significantly higher than those in SS patients (Fig. 2A). Expression of CCL18, IL-10, and IL-13 could not be detected by immunohistochemistry in SMGs from controls or CS patients, but could be detected in/around ductal epithelial cells in SS patients. In contrast, expression of CCL18, IL-10, and IL-13 was strongly detected in/around areas of fibrosis in IgG4-DS patients (Fig. 2B).

3.3. Co-localization of M2 macrophage markers and pro-fibrotic factors in SMGs from IgG4-DS patients

To clarify whether M2 macrophages expressed the pro-fibrotic factors, double immunofluorescence staining with CD163 and CCL18, IL-10 or IL-13 was performed. As shown in Fig. 3, CD163-positive cells (red) were co-localized with CCL18 and IL-10 positive cells, while they only partly co-localized with IL-13-positive cells. Therefore, M2 macrophages might promote fibrosis in SMGs from IgG4-DS patients through increased production of IL-10 and CCL18.

3.4. Evaluation of fibrosis score in SMGs

Specimens were stained by MT staining to evaluate the degree of fibrosis in SMGs. IgG4-DS patients showed severe cordlike fibrosis with extensive eGC formation, while the other groups showed only mild or moderate periductal fibrosis (Fig. 4A). The fibrosis score of IgG4-DS was significantly higher than those in the other groups (Fig. 4B). Furthermore, the fibrosis score was positively correlated with the CD163/CD68 ratio in SMGs from IgG4-DS patients but not in those from the other groups (Fig. 4C).

3.5. Expression of macrophage markers in other organs from IgG4-DS patients

To confirm the changes in macrophage distribution in IgG4-RD patients, specimens from other involved organs including the lacrimal gland, pancreas, prostate gland, and pleura from IgG4-DS patients included in this study were examined by immunohistochemical staining. Expression of

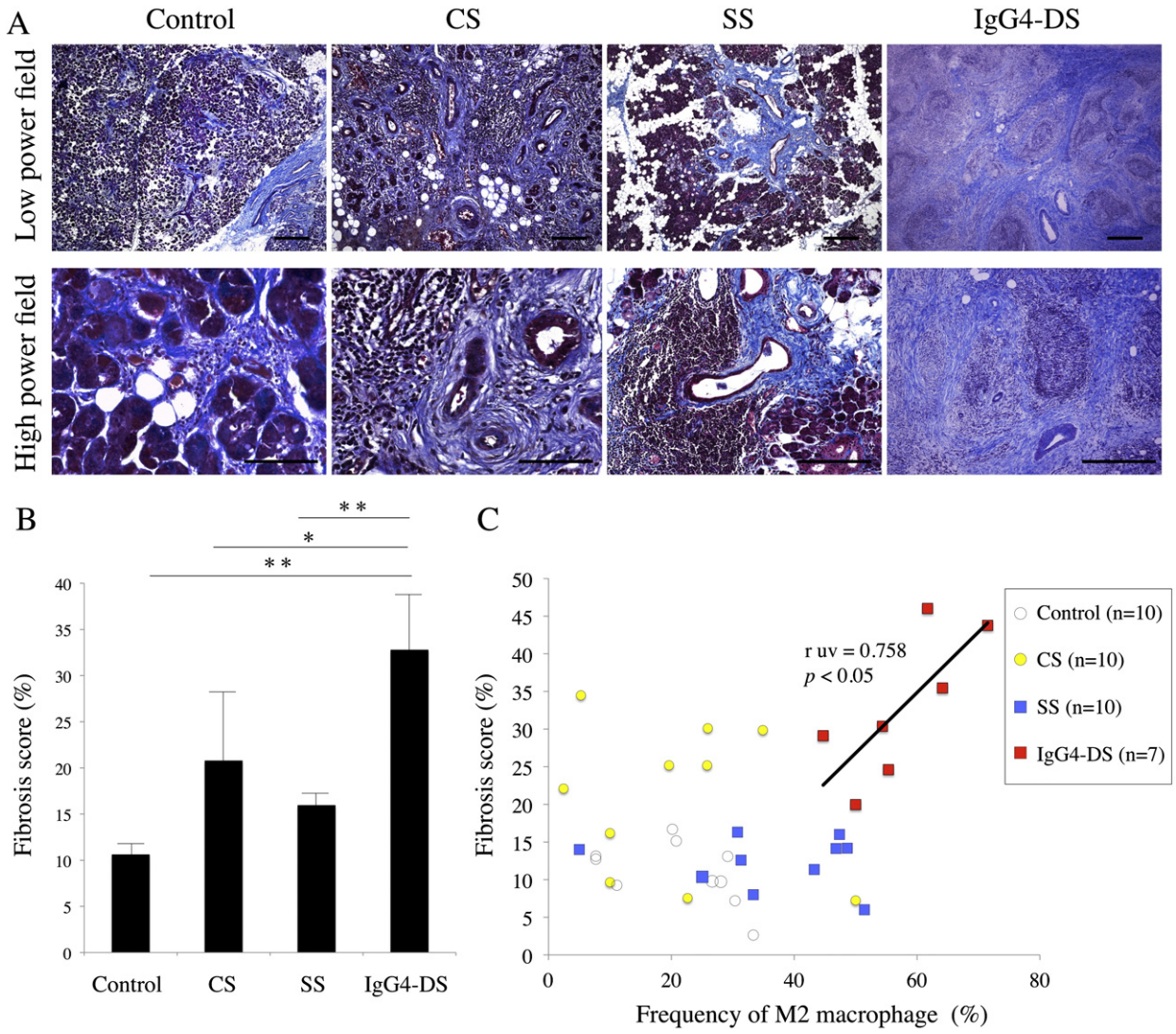

Figure 4 Correlation between M2 macrophages and fibrosis in SMGs. (A) Sections were stained by Masson's trichrome (MT) staining from controls, patients with CS, SS, and IgG4-DS. MT staining stained nuclei (purple), cytoplasm (red) and collagen (connective tissue or fibrosis) (blue) as described in the Materials and methods section. Lower magnifications are displayed in the upper line, and the higher magnifications are displayed in the lower line. Scale bars, 200 μm (lower power field) and 50 mm (high power field). (B) Evaluation of fibrosis score in the SMGs from controls (n = 10), patients with CS (n = 10), SS (n = 10), and IgG4-DS (n = 7). The fibrosis score was calculated from MT staining as described in the Materials and methods section. Statistically significant differences between groups were determined by one-way ANOVA (*$p < 0.05$, **$p < 0.01$). (C) Correlation between frequencies of M2 macrophage and fibrosis score. Statistical significance of differences between groups was determined by Spearman's rank correlation ($p < 0.05$).

CD68 and CD163 in these organs was strongly detected in/around areas of fibrosis with results similar to those obtained from SMGs (Fig. 5).

4. Discussion

IgG4-RD is now recognized as a systemic disorder, characterized by high serum IgG4, marked infiltration of IgG4-positive plasma cells and severe fibrosis with hyperplastic eGCs in swollen lesions [11]. Although our previous data revealed that Th2 adaptive immune responses induced IgG4 production and eGC formation in IgG4-RD [13,14], the mechanism of fibrosis in lesions remains to be fully elucidated. Recent studies have reported that macrophages might play a critical role in IgG4 production in IgG4-RD [15,24]. As outlined above, macrophages can be classified as M1 and M2 macrophages based on their response to the extracellular environment [25]. Notably, M2 macrophages are activated by Th2 cytokines and promote fibrosis by the production of pro-fibrotic factors (CCL18, IL-10 and IL-13) [26]. We therefore examined the M1 and M2 macrophage subsets in SMGs from SS, CS, and IgG4-DS patients. Immunohistochemical staining indicated that expression of CD163 (M2 macrophage marker) was strongly detected in IgG4-DS patients, whereas it was rarely detected in controls, CS and SS patients. These findings were mirrored

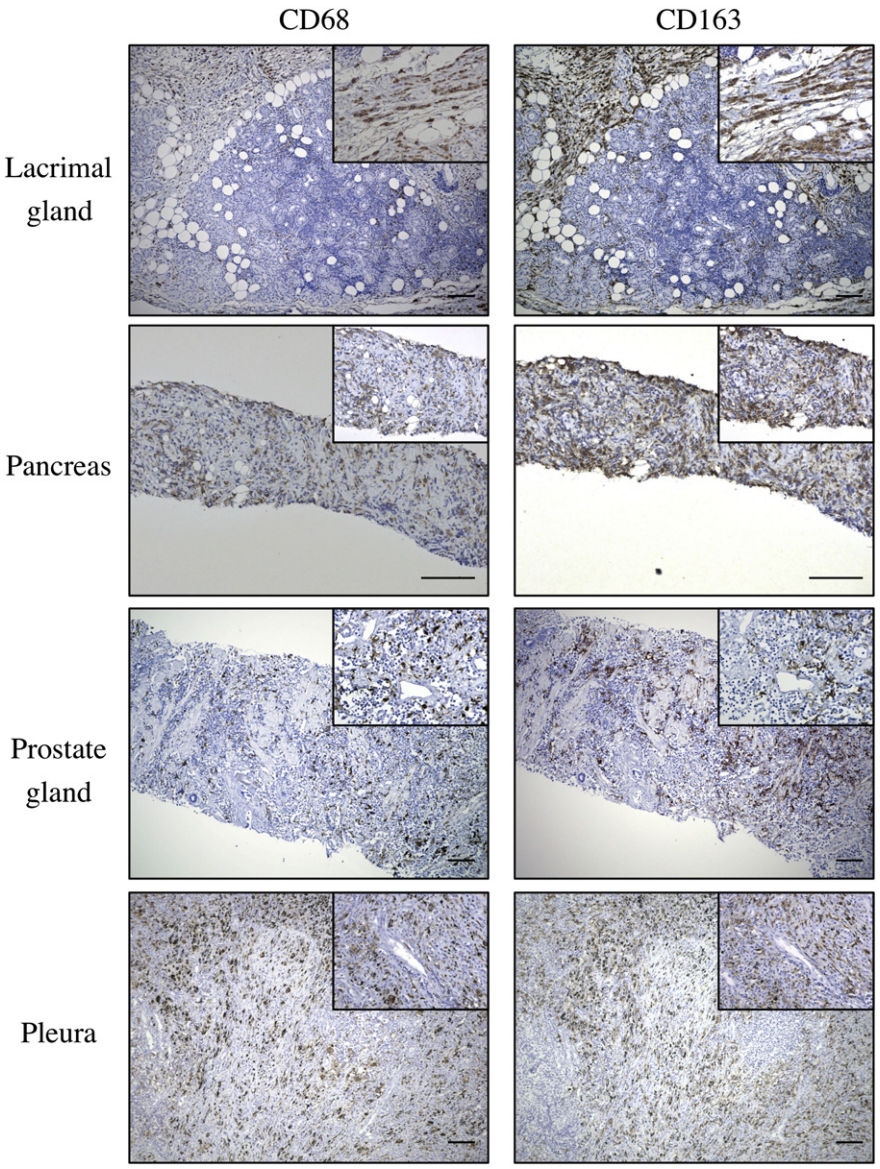

Figure 5 Distribution of M1 and M2 macrophages in the other involved organs from patients with IgG4-DS including the lacrimal gland, pancreas, prostate gland, and pleura. Counterstaining was performed with Mayer's hematoxylin (blue). The higher magnifications are displayed at the upper right. Scale bars, 100 μm.

in other lesions including the lacrimal gland, pancreas, pleura, and prostate gland from IgG4-RD patients. Interestingly, the cell number and frequency of M2 macrophage in IgG4-DS were significantly higher than those in the other groups. To reveal the relationship between M2 macrophages and fibrosis in SMGs from IgG4-DS patients, we also analyzed the expression of pro-fibrotic factors such as CCL18, IL-10, and IL-13. The expression patterns of CCL18 and IL-10 in SMGs were similar to those of M2 macrophages in patients with IgG4-DS. In contrast, IL-13 was mainly expressed on other cells such as Th2 and mast cells in patients with IgG4-DS. In this study the mRNA expression levels of CD163 and pro-fibrotic factors (CCL18, IL-10, and IL-13) in SMGs from patients with IgG4-DS were higher than those in the other groups. Furthermore, the fibrosis scores were positively correlated with the frequency of M2 macrophages only in patients with IgG4-DS. These results suggest that preferential M2 macrophages might be involved in fibrosis of swollen lesions from IgG4-RD patients through the production of CCL18 and IL-10. On the other hands, Th2 and mast cells might promote fibrosis through the production of IL-13 without involving M2 macrophages.

IL-10 is well known as an immunosuppressive cytokine, produced mostly by macrophages, Th2 cells, regulatory T cells (Tregs) and dendritic cells (DCs), and is essential for the maintenance of immunological self-tolerance and immune homeostasis. Several studies have reported that IL-4 and IL-10 are overexpressed locally in the lesions of IgG4-RD patients and contribute to IgG4-specific class switching [13,27,28]. With regard to fibrosis, IL-10 was identified as an endogenous inhibitor of tissue fibrosis [29,30], while recent studies demonstrated that IL-10 produced by M2

macrophage had an important role in the renal and lung fibrosis [31,32]. In this study, the localization of IL-10 in SMGs from IgG4-DS patients was consistent with that of production by M2 macrophages, suggesting that the majority of IL-10 producing cells might be M2 macrophages and deeply involved in fibrosis. In contrast, other IL-10 producing cells such as Treg and plasmacytoid DC were also infiltrated in salivary glands from IgG4-DS patients. However, these cells were detected in/around GCs rather than around fibrotic lesions, and might promote plasma cells to IgG4 production (manuscript in preparation).

CCL18 is one of the most highly expressed chemokines in chronic inflammatory diseases including idiopathic pulmonary fibrosis [33], bronchial asthma [34] and atopic dermatitis [35]. In addition, CCL18 is now recognized as a chemokine with not only fibrotic activity but also selective chemotactic activity on peripheral blood T lymphocytes, especially Th2 cells [36,37]. Although IgG4-RD is considered a Th2-dominant disease [12], the mechanism of Th2 polarization has yet to be elucidated. Considering the current results, M2 macrophages might also contribute to Th2 polarization of IgG4-RD by CCL18 production. However, additional research is required to further elucidate the involvement of innate immunity in the pathogenesis of IgG4-RD.

Fibrosis associated with a part of IgG4-RD including AIP, IgG4-SC and IgG4-TIN has a characteristic irregular whorled pattern, termed "storiform fibrosis" [19]. In this study, we have confirmed that the production of IL-10 and CCL18 by preferential M2 macrophages is associated with severe fibrosis in IgG4-RD. However, the relationship between M2 macrophage and this fibrotic pattern in salivary glands was not distinctive change, and requires more consideration for other organs. Recent studies reported that rituximab targeting peripheral CD20-positive plasma cells appeared to be an effective treatment strategy for IgG4-RD [38,39]. In addition, circulating plasmablasts, derived from the B cell lineage, are also elevated in IgG4-RD patients, especially with resistance to rituximab [40,41]. This is attributed to the fact that plasmablasts lack surface expression of CD20 and demonstrate a resistance to direct depletion by rituximab. Therefore, IgG4-positive plasmablasts are considered to be prominently involved in the pathogenesis of IgG4-RD. Interestingly, after interacting with antigens exposed on macrophages, marginal zone B cells rapidly differentiate into plasmablasts [42]. These results suggest that macrophages might play an effective role in plasmablast activation of IgG4-RD. A more thorough understanding of the immune mechanism of IgG4-RD, especially the role of innate immune cells, could lead to the development of novel pharmacological strategies aimed at disrupting the recruitment of inflammatory cells to the local lesion and inhibiting the initiation of IgG4-RD.

Conflict of interest

The authors declare no competing interests.

Author contributions

All authors provided substantial contributions to discussions of content, and to reviewing and editing the manuscript before submission. M Moriyama researched the data and wrote the article.

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